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Published in:
EBioMedicine

DOI:
[10.1016/j.ebiom.2019.07.057](https://doi.org/10.1016/j.ebiom.2019.07.057)

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Rago, D., Rasmussen, M. A., Lee-Sarwar, K. A., Weiss, S. T., Lasky-Su, J., Stokholm, J., Bønnelykke, K., Chawes, B. L., & Bisgaard, H. (2019). Fish-oil supplementation in pregnancy, child metabolomics and asthma risk. *EBioMedicine*, 46, 399-410. <https://doi.org/10.1016/j.ebiom.2019.07.057>



Research paper

Fish-oil supplementation in pregnancy, child metabolomics and asthma risk



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ARTICLE INFO

Article history:

Received 17 May 2019

Received in revised form 22 July 2019

Accepted 22 July 2019

Available online 6 August 2019

Keywords:

Metabolomics

Fish oil

Childhood asthma

ABSTRACT

Background: We recently demonstrated that maternal dietary supplementation with fish oil-derived n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) during pregnancy reduces the risk of asthma in the offspring but the mechanisms involved are unknown.

Methods: Here we investigated potential metabolic mechanisms using untargeted liquid chromatography-mass spectrometry-based metabolomics on 577 plasma samples collected at age 6 months in the offspring of mothers participating in the n-3 LCPUFA randomized controlled trial. First, associations between the n-3 LCPUFA supplementation groups and child metabolite levels were investigated using univariate regression models and data-driven partial least square discriminant analyses (PLS-DA). Second, we analyzed the association between the n-3 LCPUFA metabolomic profile and asthma development using Cox-regression. Third, we conducted mediation analyses to investigate whether the protective effect of n-3 LCPUFA on asthma was mediated via the metabolome.

Findings: The univariate analyses and the PLS-DA showed that maternal fish oil supplementation affected the child's metabolome, especially with lower levels of the n-6 LCPUFA pathway-related metabolites and saturated and monounsaturated long-chain fatty acids-containing compounds, lower levels of metabolites of the tryptophan pathway, and higher levels of metabolites in the tyrosine and glutamic acid pathway. This fish oil-related metabolic profile at age 6 months was significantly associated with a reduced risk of asthma by age 5 and the metabolic profile explained 24% of the observed asthma-protective effect in the mediation analysis.

Interpretation: Several of the observed pathways may be involved in the asthma-protective effect of maternal n-3 LCPUFA supplementation and act as mediators between the intervention and disease development.

Funding: COPSAC is funded by private and public research funds all listed on www.copsac.com.

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1. Introduction

Asthma is the most common chronic disease in childhood and has doubled in prevalence over the last decades in Westernized countries [1]. This rapid increase in disease prevalence is believed to be caused by changes in lifestyle and human diet, particularly, an increased intake

of n-6 long-chain polyunsaturated fatty acids (n-6 LCPUFAs) and a decreased intake of n-3 LCPUFAs among pregnant mothers, has been associated with an increased risk of asthma in the offspring [2]. The main dietary source of n-3 LCPUFA comes from fish and other seafood, especially from cold-water fatty fish such as salmon and mackerel, and/or from dietary supplements such as dietary fish oil capsules. Due to those observations, we conducted a double-blind randomized controlled trial (DB-RCT) of supplementation with fish oil during pregnancy in the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC₂₀₁₀) mother-child cohort. This resulted in a 31% reduced risk of asthma in the offspring during their first 5 years of life [3]. Thus, n-3 LCPUFA supplementation during pregnancy holds promise for the primary prevention of childhood asthma, but the underlying mechanisms of the asthma-protective effect of LCPUFA remain to be elucidated.

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Research in context

Evidence before this study

The global increase in childhood asthma prevalence has been observed in parallel with an increasing dietary intake of n-6 polyunsaturated fatty acids (PUFA) from e.g. vegetable oils and decreasing intake of n-3 LCPUFAs found in fish, suggesting a modifiable link between n-3 LCPUFA deprived diet during pregnancy and offspring asthma risk. Recently, we proved that hypothesis in a double-blind, randomized controlled trial of supplementation with fish oil-derived n-3 LCPUFA or placebo during third pregnancy trimester, showing 31% reduced risk of asthma in the offspring by age 5. However, the underlying mechanism behind the supplementation effect is unknown.

Added value of this study

In this study we used global, untargeted metabolomics profiles from plasma samples collected at age 6 months in the children born to mothers participating in the n-3 LCPUFA trial to investigate metabolic perturbations related to the prenatal n-3 LCPUFA supplementation and whether those perturbations explained some of the underlying biology of the asthma-protective effect. We observed differences in several metabolic pathways, which have previously been related to asthma such as n-6 LCPUFA pathway-related metabolites and tryptophan metabolites as well as metabolites in the tyrosine and glutamic acid pathway. Further, we showed that these n-3 LCPUFA related metabolic perturbations were associated with a reduced risk of asthma by age 5 and significantly mediated one fourth of the asthma-protective effect observed in the trial.

Implications of all the available evidence

These findings provide novel insight into the metabolic effects of dietary n-3 LCPUFA supplementation and the pathogenesis of childhood asthma. Further, this study is an example of translational research, where mechanisms of a clinically important finding from a dietary, randomized controlled trial are explored using metabolomics.

Untargeted metabolomics provides a global assessment of the metabolome, which is the complete set of small-molecule metabolites in a biological sample. By generating a complete metabolomic profile of a specific biofluid, such as plasma, from a cohort of individuals [4], metabolomics can be applied to disentangle molecular mechanisms of complex diseases such as asthma or metabolic consequences of environmental exposures, such as diet. Hitherto, metabolomic profiling studies have shown promising results for identifying the underlying mechanisms of asthma [5]. Furthermore, metabolomics enables studying the long-term downstream metabolic effects in the child of micronutrient and macronutrient supplementation during pregnancy [6], such as n-3 LCPUFAs.

The objective of this study was to conduct plasma metabolomic profiling using untargeted liquid chromatography-mass spectrometry (LC-MS) technique on blood sampled at age 6 months in the COPSAC₂₀₁₀ cohort to explore potential biochemical mechanisms behind the asthma-protective effect of n-3 LCPUFA supplementation during pregnancy.

2. Methods

2.1. Study population

The Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀ (COPSAC₂₀₁₀) cohort is a population-based mother-child cohort in which 700 pregnant women were recruited between 22 and 26 weeks

of gestation and their children subsequently followed prospectively during their first five years of life at 12 scheduled clinical visits. All the visits were conducted at the COPSAC research center, where trained pediatricians examined the children and collected exposure information at age 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months after birth, and yearly thereafter. In addition, whenever the children experienced lung, allergy or skin-related symptoms the families attended acute care visits at the research unit, where the COPSAC pediatricians diagnosed and treated asthma, allergy, and eczema in accordance with predefined validated algorithms [7]. Furthermore, the parents filled daily diary cards prospectively from birth recording significant troublesome lung symptoms, including cough, wheeze, and dyspnea, anti-asthmatic treatment, skin symptoms and treatment, and infections.

2.2. n-3 LCPUFA trial

The pregnant women were enrolled in a double-blind, randomized controlled trial (DB-RCT) [3]. At enrollment during pregnancy week 22–26, the women were randomly assigned in a 1:1 ratio to receive 2.4 g per day of n – 3 long-chain polyunsaturated fatty acids (LCPUFAs) in triacylglycerol form from fish oil capsules containing 55% eicosapentaenoic acid (20:5n–3, EPA) and 37% docosahexaenoic acid (22:6n–3, DHA (Incomomega TG33/22, Croda, Health Care) or placebo in the form of lookalike olive oil capsules, containing 72% n–9 oleic acid and 12% n – 6 linoleic acid (Pharma-Tech A/S). The women continued taking the daily supplement until 1 week after delivery, where they visited the research unit with their child. A subgroup of the pregnant women ($n = 623$) also participated in another DB-RCT with a nested 2×2 factorial design in which they were assigned to 2400 IU of vitamin D per day or placebo from pregnancy week 22–26 till 1 week postpartum on top of the recommended pregnancy supplement of 400 IU/d; i.e. the women received 2800 vs. 400 IU/d of vitamin D, which did not significantly affect the offspring's risk of developing asthma [3].

2.3. Ethics statement

The trial was approved by The National Committee on Health Research Ethics (H-B-2008-093) and the Danish Data Protection Agency (2015-41-3696). Both parents gave oral and written informed consent before enrollment.

2.4. Asthma diagnosis

Asthma or persistent wheeze was the primary end point of the DB-RCT of n-3 LCPUFA supplementation during pregnancy and was solely diagnosed by the COPSAC pediatricians based on a quantitative validated symptom algorithm [8,9], including all of the following four criteria: (1) verified diary recordings of at least five episodes of troublesome lung symptoms within six months, each lasting at least three consecutive days; (2) symptoms typical of asthma, including exercise-induced symptoms, prolonged nocturnal cough, and/or persistent cough outside of common colds; (3) need for intermittent rescue use of inhaled β_2 -agonist; and (4) response to a three-month course of inhaled corticosteroids and relapse upon ending treatment [8].

2.5. Covariates

2.5.1. Breastfeeding

Information about the duration of exclusive and total breastfeeding duration was obtained during the scheduled visits to the COPSAC clinic and recorded online in a dedicated database.

2.5.2. FADS genotyping

Maternal and child variation in the gene encoding fatty acid desaturase (FADS2) was assessed by genotyping the single-nucleotide polymorphism (SNP) rs1535. Genotyping was performed using the

Illumina Infinium HumanOmniExpressExome Bead chip, at the AROS Applied Biotechnology AS center, Aarhus, Denmark. Genotypes were called with Illumina Genome Studio software and rs1535 was genotyped on this array. We excluded individuals with individual genotyping call rate < 0.95, gender mismatch, genetic duplicates or outlying heterozygosity > 0.27 and < 0.037. Furthermore, maternal genotyping was solely done in mothers of European descent as there are very few non-Caucasian mothers in the cohort.

2.5.3. Blood sample collection and storage

A blood sample was collected from the child when visiting the research clinic at age 6 months. The blood sample was collected in an EDTA tube and left at room temperature for 30 min and thereafter spun down for 10 min at 4000 rpm. The supernatant was collected and stored at -80°C until further analysis.

2.6. Liquid Chromatography-Mass Spectrometry (LC-MS) metabolomics analysis

2.6.1. Sample preparation

The untargeted metabolomic analysis of the plasma samples was carried out by Metabolon, Inc. (NC, USA). Briefly, the sample preparation was done using the automated system MicroLab STAR® system from Hamilton Company. Prior to extraction, the samples were fortified with recovery standards for quality control (QC) purposes. Methanol was used to extract the metabolites and precipitate the proteins during vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into four aliquots to be subsequently analyzed by LC-MS/MS comprising of four platforms. Samples were placed on a TurboVap® (Zymark) to remove the organic solvent and stored overnight under nitrogen before preparation for LC-MS/MS analysis.

2.6.2. LC-MS/MS analysis

All the four platforms utilized an ACQUITY Ultra-Performance Liquid Chromatography (UPLC) (Waters, Milford, USA) and a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer interfaced with heated electrospray ionization (HESI-II) source (ThermoFisher Scientific, Waltham, Massachusetts, USA). The sample extracts were reconstituted in solvents compatible to each of the four LC-MS methods: two separated reverse phase UPLC-ESI(+)MS/MS methods optimized for hydrophilic and hydrophobic compounds; one reverse phase UPLC-(−)MS/MS using basic optimized conditions; and one HILIC/UPLC-(−)MS/MS. The MS analysis alternated between full scan MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range for both ionization modes was 70–1000 m/z.

2.6.3. Data collection and quality control

The raw data was extracted, peak identified, and QC processed. Samples were semi-quantified using area-under-the-curve. Peak identification was done by automated comparison of the ion feature in the experimental spectrum to the ions present in the library spectrum of authenticated standards or recurrent unknown entities. The identification was based on three matching criteria: narrow retention time/index (RI) range, mass accuracy (± 10 ppm) and MS/MS spectra. The identification level reported in this paper follows the criteria described by Summer LW et al. [10]. Compounds labelled with “*” have identification level 2, compounds labelled with “***” have level 3 (since no standards or matching spectra are available). Compounds named with “X-#” are unknown and therefore have level 4. If no label is applied, the identification level is 1.

Throughout the analyses of the batches, different kinds of samples were analyzed for instrument performance monitoring: a well-characterized human plasma as a technical replicate, extracted water samples as process blanks, and a mixture of QC standards (not interfering with the measurement of endogenous compounds) spiked in every

analyzed sample prior to injection. The instrument variability was determined by calculating the median relative standard deviation (RSD) for the QC standards spiked in. It ranged around 7%. The total process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in the technical replicates. It resulted to be within 10%.

2.7. Statistical analysis

2.7.1. Data preprocessing

Each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each variable, accordingly. This data normalization procedure that was done for all the dataset from the four platforms was performed to compensate for the inter-day variation. The final set of variables from all the four platforms was imported into Matlab (Version 9.3, the Mathworks, Inc., MA, USA) and RStudio (Version 1.1, RStudio, Inc) for the statistical analysis.

In order to remove spurious information prior to data analysis, samples having $\geq 30\%$ of missing values as well as metabolites containing $\geq 70\%$ of missing values were discarded. Furthermore, since the aim of our analysis was to investigate the effect of the n-3 LCPUFA supplementation during pregnancy on the child metabolome at 6 months of age, variables not present in at least 70% of the samples in either the n-3 LCPUFA or placebo group were removed [11]. Finally, missing values were imputed with zeros.

2.7.2. Univariate linear regression analysis

Firstly, linear regression analysis was performed to compare the relative concentrations of the imported set of metabolites (features) in the n-3 LCPUFA supplementation vs. placebo group. Significant features were reported at a nominal significance level in this exploratory analysis (p -values $\leq .05$ from “Wald test”).

Linear regression analysis was also used to correlate solely breastfeeding duration with selected metabolite levels. The intercept of such model also allowed to estimate the levels of the compounds independent by the breastfeeding. Thereafter, stratifying the analysis by the n-3 LCPUFA intervention allowed us to estimate the difference in modelled metabolite levels at birth vs. at 6 months (time of sampling) by the slope. In order to test if those differences were significant, we used simultaneous tests for general linear hypotheses from the “multcomp” R package.

Influence of maternal and child FADS2 genotype were also investigated using linear regression models. Genotype was associated with selected metabolites level in the n-6 pathway stratified by treatment and accounting for each other genotype and exclusively breastfeeding duration. The association is reported per copy of the minor allele G in terms of beta-estimate, standard error (SE) and significance (p -value from “Wald test”).

2.7.3. Multivariate partial least squares discriminant analysis (PLS-DA)

After the univariate analysis, PLS-DA was employed to classify the differences between metabolites in the n-3 LC-PUFA supplement group compared to placebo. Initially, the dataset was imported into the PLS_Toolbox (Version 8.61, Eigenvector Research, Inc., MA, USA) and auto-scaled prior to analysis. The PLS-DA model validation was done by iterating 100×, using a 10-fold cross-validation model in the training set in which the number of PLS-DA components was chosen based on the lowest misclassification error. During each iteration, the rank of each feature was recorded based on its selectivity ratio (SR) and the model was validated based on misclassification error on a random test set selected up-front and using 20% of the original dataset. The product of the ranks was used to sort the variables ascending (low rank, high importance) and successively to select the most important ones based on the lowest misclassification error on the random test set (20% of the total samples) when models were built including 10 variables at the time and starting with 11 initial features [12]. Furthermore,

a permutation test using 100× iterations was applied to assess the classification performance of the final PLS-DA model with the selected variables, where the *p*-values are from a randomization *t*-test.

2.7.4. Rotated model

The PLS-DA model estimates the linear combination of the original matrix (\mathbf{X} (n, p))) that most optimally relates to the intervention (\mathbf{f} ($n, 1$))) by finding a so called score matrix ($\mathbf{T} = \mathbf{XW}$ (n, k))). This score matrix by definition captures the structure in the metabolomics data table (\mathbf{X}) that has maximum covariance with the intervention. However, as the estimation do not utilize information of the clinical outcome (asthma at 6y of age: \mathbf{o} ($n, 1$))), the metabolomics intervention fingerprint related to asthma will be distributed across all *k* components. In order to make a more parsimonious model, that in the first component (\mathbf{T}_1) reflects the intervention fingerprint related to outcome while leaving the part that is only related to intervention, but not outcome in the remaining components ($\mathbf{T}_2, \dots, \mathbf{T}_k$) the score matrix is rotated towards the outcome using orthogonal procrustes rotation [13].

2.7.5. Enrichment analysis

One-sided Fisher's exact test was performed to investigate for any pathway enrichment within the selected metabolites. For each pathway, a contingency table (2×2) was made, see Table S1 in the Supplementary Material for further details. Enrichment is defined for significantly (*p*-value < .05) higher odds for inclusion of a metabolite for the *i*'th pathway compared to all other pathways.

2.7.6. Cox-regression and mediation analysis

Cox regression analysis using the “survival” R package was employed to assess the correlation between the components of the rotated PLS-DA model (which were z-scored), that describes the difference between n-3 LCPUFA and placebo, and the risk of developing asthma during the first 5 years of life. Reported *p*-values are from Wald test. A Kaplan-Meier curve was used to representing the association between the PLS-DA component scores divided into tertiles and risk of developing asthma.

To assess the mediation effect between the components of the rotated PLS-DA model and the development of asthma during the first 5 years of life, the R package “mediation” was used with 95% CIs based on quasi-Bayesian approximation using 10,000 Monte-Carlo draws. The mediation analysis was built in the following way:

firstly, a linear model was built associating the rsLV1 and the n-3 LCPUFA supplementation (Model 1), followed by a binomial model associating the asthma diagnosed till age 5 years ($n = 116$) and the n-3 LCPUFA supplementation plus the rsLV1 (Model 2). Afterwards, a third model was built combining these two models together and imposing rsLV1 as mediating factor between the asthma development and the n-3 LCPUFA supplementation (Model 3).

Model 1 (Intervention vs rLV1) : $\text{lm}(\text{rsLV1} \sim \text{intervention})$ (1)

Model 2(asthma) : $\text{glm}(\text{asthma} \sim \text{intervention} + \text{rLV1})$ (2)

Model 3(Model 1 + Model 2)
: $\text{mediation}(\text{Model 1, Model 2, mediator} = \text{“rLV1”})$

2.8. Data availability

De-identified metabolomics data is deposited in the publicly available data repository, “Metabolomics Workbench” under Study ID: **ST001212**, and it is also available with this article (Supplementary material 1).

3. Results

3.1. Baseline characteristics of the study

The COPSAC₂₀₁₀ mother-child cohort of 736 pregnant women and their 695 children has previously been described in detail [7]. Blood samples were collected for metabolomic profiling from 602 children at 6 months up to 2 years of age. Of those, only samples from children aged 4–8 months were included in the analysis as their metabolomic profiles were similar in a principal component analysis (PCA) compared to samples collected from children at older ages (see Supplementary Material). These metabolomic profiles contained 1138 unique metabolites that were identified using mass to charge ratio, retention time, and a library of metabolites (see Supplementary Material). Following the subsequent quality control steps removing samples with ≥30% missing values as well as features with ≥70% missing values resulted in a final dataset including samples from 577 children (51% boys) with 831 metabolites. The composition of the remaining metabolites after these quality control procedures is shown in Figure SF3 (in the Supplementary Material), where the metabolites are grouped based on sub pathway (Panel a) and the most abundant pathways with >10 compounds, which are lipids and amino acids (Panel b).

A total of 51 (8.9%) children were exclusively breastfed for less than a week from birth, 553 (96.2%) terminated the exclusive breastfeeding before the time of metabolomic sampling, whereas 22 (3.8%) were still exclusively breastfed at the time of metabolomic sampling, and breastfeeding status was unknown for 2 children. An overview of the baseline characteristics is presented in Table S2 (see Supplementary Material).

3.2. n-3 LCPUFA supplementation during pregnancy and the child metabolome

In order to investigate differences in the plasma metabolomic profiles between children whose mothers received n-3 LCPUFA supplementation or placebo, we used univariate linear regression models for each metabolite followed by a partial least squares discriminant analysis (PLS-DA) model.

3.3. Univariate linear regression analysis

Among the 831 features investigated, 42 showed a nominally significant difference at a $p = .05$ level between the n-3 LCPUFA supplementation and the placebo group. The volcano plot in Figure SF5 [in the Supplementary Material] summarizes the results showing the effect estimates vs. *p*-values. The results are further detailed in the heatmap in Figure SF6 [in the Supplementary Material] that shows the significant metabolites and their associated sub-pathways. As shown in Figure SF6, the lipids and the amino acid pathways are the ones most affected by the n-3 LCPUFA supplementation with higher hydroxy-3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (hydroxy-CMPF) and lower n-6 docosapentaenoic acid (DPA, 22:5n-6) being the two most perturbed compounds. Among the significant metabolites, 7 have unknown identities, see Table S3 [in the Supplementary Material] and will not be mentioned or discussed further. Due to natural collinearity between metabolites and the scope of the univariate models being merely exploratory supportive analyses, we did not apply FDR correction. However, the univariate analyses results should be interpreted with caution.

3.4. Multivariate PLS-DA and pathway enrichment analysis

Based on all the metabolome features for classifying children according to pregnancy supplementation, the best PLS-DA model comprised 5 components and 121 metabolites. This model had a misclassification error of 0.29, an AUC of 0.77 and a permutation test *p*-value = .005

for discriminating between n-3 LCPUFA supplementation and placebo. The score plot of the first two components (LV1 and LV2, explaining 10.99% and 9.39% of the variation, respectively) is shown in Fig. 1 Panel A, illustrating that children in the n-3 LCPUFA supplementation group have higher scores in both components compared to children in the placebo group. The corresponding loading plot is shown in Fig. 1 Panel a, where the significant compounds from the univariate linear regression analysis are labelled.

Most of the observed association of the pregnancy n-3 LCPUFA supplementation on the child metabolome is related to lower levels of lipid-related compounds containing n-6 unsaturated, monounsaturated or saturated long-chain fatty acids (FAs). Especially, arachidonic acid (AA, 20:4n6) and its esterified forms (plasmalogens, lysophosphatidylcholines, phosphatidylcholines, phosphatidylinositols, diacylglycerols), dihomolinolenic acid (20:3n-6) and n-6 docosapentaenoic acid (n6-DPA, 22:5n-6) were lower in the n-3 LCPUFA supplementation group. This is illustrated in Table 1, which provides an overview of the metabolites contributing the most in the first two PLS-DA components grouped by sub-pathway, including the fold change. For the remaining set of metabolites ($N = 69$), see Table S5 in the Supplementary Material.

The n-3 LCPUFA supplementation also resulted in a decrease in xanthurenic acid in the tryptophan pathway and an increase in the glutamic acid and tyrosine pathway-related compounds. We also observed association with the carnitine metabolism pathway with increase of carnitines and decrease of acylcarnitine-related compounds. Finally, a decrease of ceramides and sphingolipids containing 18:0 and 22:0 FAs and an increase in hydroxy-CMPF, a metabolite associated with fish or fish oil intake, was prevalent in the children, whose mothers received n-3 LCPUFA (Table 1).

Among the 121 metabolites in the final PLS-DA model, 29 compounds are of unknown identity and are reported in Table S5 in the Supplementary Material. Performing an enrichment analysis based on sub-pathways on the remaining 92 metabolites, we observed significant enrichment in the tyrosine pathway, as shown in Fig. 2.

3.5. Maternal and child FADS2 genotype and LCPUFA levels at age 6 month

It has been shown that levels of n-3 LCPUFAs are related to the activity of the fatty acid desaturase (FADS) enzymes ($\Delta 5$ -desaturase and $\Delta 6$ -desaturase), which are involved in the formation of the LCPUFAs from essential FAs [14]. Those enzymes are encoded by the FADS1 and FADS2 genes located on chromosome 11 [15,16].

In this DB-RCT of n-3 LCPUFA supplementation during pregnancy, we showed that the mother's levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at enrollment at pregnancy week 24 were associated with the FADS2 genotype (rs1535) with lower levels in mothers carrying the minor allele (G) [3]. Therefore, we tested whether maternal and/or child FADS2 genotype influenced the levels of the FAs and esterified forms in the n-6 pathway, that we found were significantly associated with the n-3 LCPUFA supplementation in our analysis. The analyses were stratified by n-3 LCPUFA supplementation or placebo using both maternal and child FADS2 genotype in models adjusted for each other. The results are presented in Table 2 showing that maternal FADS2 genotype was significantly and negatively associated with the levels of all the n-6 pathway-related metabolites in the placebo strata with lower levels per number of the G minor allele, whereas no associations were observed for maternal FADS2 genotype in the n-3 LCPUFA group. No associations were observed between child FADS2 genotype and the n-6 pathway-related metabolites in either the n-3 LCPUFA or the placebo strata.

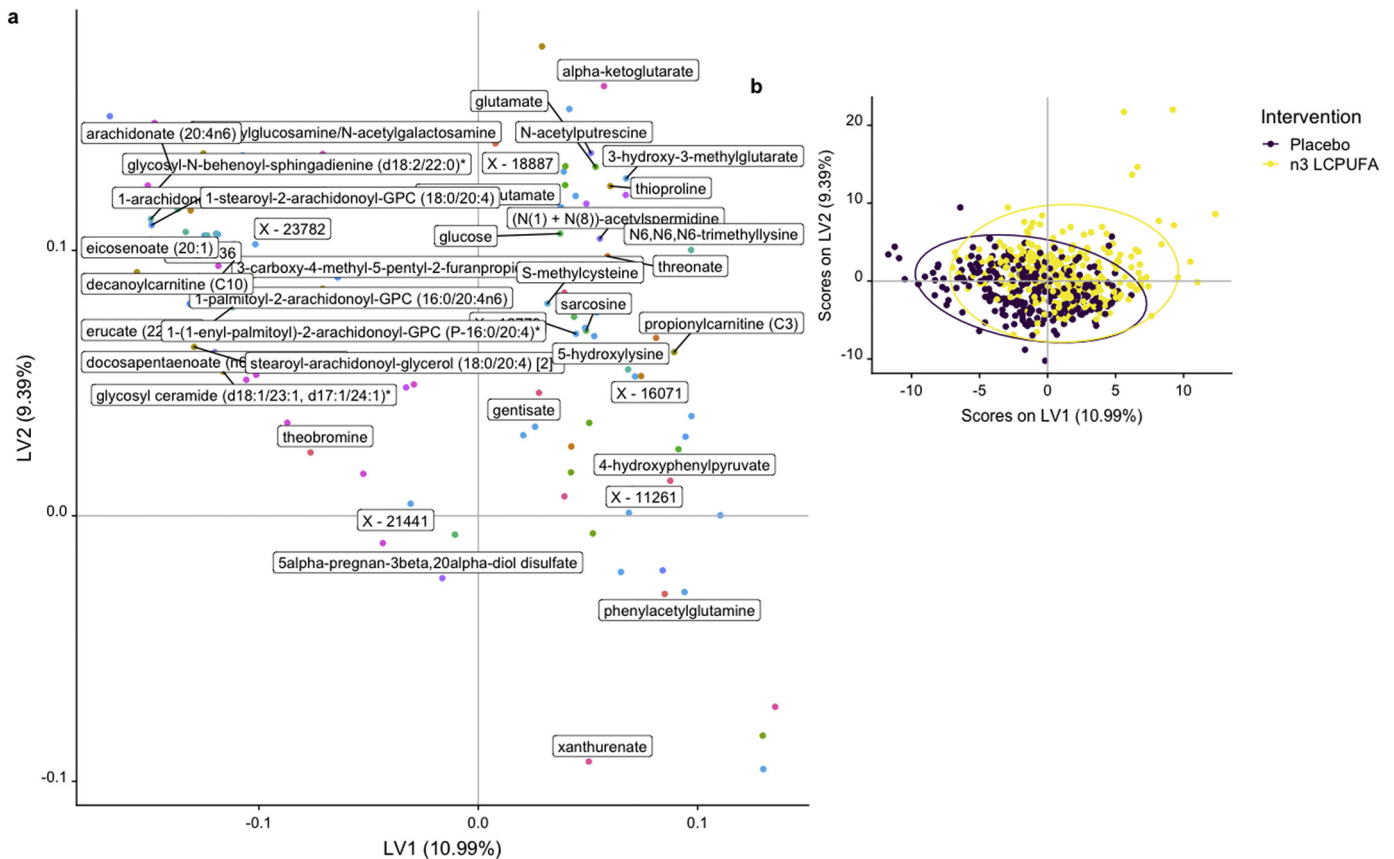


Fig. 1. PLS-DA analysis. a) Loadings plot of the first two components of the PLS-DA analysis. The compounds are colored based on their sub-pathway. The labelled compounds are the ones in common with the linear regression analysis. b) Scores plot of the first two components of the PLS-DA analysis. The samples are colored based on the intervention (n-3 LCPUFA or placebo).

Table 1
Main metabolites influencing the PLS-DA model, reported in terms of effect size when stratifying by the intervention. A negative effect size represents the effect towards the placebo group.

Compound, Sub-pathway	Treatment	Effect size	Metabolism
1-(1-Enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, Plasmalogen	Placebo n3 LCPUFA	−0.2	n3/n6 Fatty Acid Metabolism
1-(1-Enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*, Plasmalogen	Placebo n3 LCPUFA	−0.1	
1-Arachidonoyl-GPC (20:4n6)*, Lysophospholipid	Placebo n3 LCPUFA	−0.2	
1-Arachidonoyl-GPE (20:4n6)*, Lysophospholipid	Placebo n3 LCPUFA	−0.2	
1-Palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6), Phosphatidylcholine (PC)	Placebo n3 LCPUFA	−0.2	
1-Stearoyl-2-arachidonoyl-GPC (18:0/20:4), Phosphatidylcholine (PC)	Placebo n3 LCPUFA	−0.2	
1-Stearoyl-2-arachidonoyl-GPI (18:0/20:4), Phosphatidylinositol (PI)	Placebo n3 LCPUFA	−0.2	
Dihomo-linolenate (20:3n3 or n6), Polyunsaturated Fatty Acid (n3 and n6)	Placebo n3 LCPUFA	−0.1	
Docosapentaenoate (n6 DPA; 22:5n6), Polyunsaturated Fatty Acid (n3 and n6)	Placebo n3 LCPUFA	−0.3	
Palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]*, Diacylglycerol	Placebo n3 LCPUFA	−0.1	
Stearidonate (18:4n3), Polyunsaturated Fatty Acid (n3 and n6)	Placebo n3 LCPUFA	−0.2	Lipid oxidation
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [1]*, Diacylglycerol	Placebo n3 LCPUFA	−0.2	
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [2]*, Diacylglycerol	Placebo n3 LCPUFA	−0.2	
3-Hydroxybutyrate (BHBA), Ketone Bodies	Placebo n3 LCPUFA	−0.1	
Carnitine, Carnitine Metabolism	Placebo n3 LCPUFA	0.1	
Decanoylcarnitine (C10), Fatty Acid Metabolism(Acyl Carnitine)	Placebo n3 LCPUFA	−0.2	
Myristoleoylcarnitine (C14:1)*, Fatty Acid Metabolism(Acyl Carnitine)	Placebo n3 LCPUFA	−0.2	
Octanoylcarnitine (C8), Fatty Acid Metabolism(Acyl Carnitine)	Placebo n3 LCPUFA	−0.2	
Propionylcarnitine (C3), Fatty Acid Metabolism (also BCAA Metabolism)	Placebo n3 LCPUFA	0.2	
3-(4-Hydroxyphenyl)lactate, Tyrosine Metabolism	Placebo n3 LCPUFA	0.1	Tyrosine Metabolism
3-Methoxytyramine sulfate, Tyrosine Metabolism	Placebo n3 LCPUFA	0.1	
4-Hydroxyphenylpyruvate, Tyrosine Metabolism	Placebo n3 LCPUFA	0.2	
Gentisate, Tyrosine Metabolism	Placebo n3 LCPUFA	0.2	
Tyrosine, Tyrosine Metabolism	Placebo n3 LCPUFA	0.1	
Vanillylmandelate (VMA), Tyrosine Metabolism	Placebo n3 LCPUFA	0.1	
4-Hydroxyglutamate, Glutamate Metabolism	Placebo n3 LCPUFA	0.3	Glutamate Metabolism
Glutamate, Glutamate Metabolism	Placebo n3 LCPUFA	0.2	
3-Indoxyl sulfate, Tryptophan Metabolism	Placebo n3 LCPUFA	0.1	Tryptophan Metabolism
Xanthurenate, Tryptophan Metabolism	Placebo n3 LCPUFA	−0.2	
3-Carboxy-4-methyl-5-pentyl-2-furanpropionate (3-Cmpfp)**, Fatty Acid, Dicarboxylate	Placebo n3 LCPUFA	−0.2	Furan Fatty Acid Metabolism
Hydroxy-CMPF*, Fatty Acid, Dicarboxylate	Placebo n3 LCPUFA	0.8	
N-Behenoyl-sphingadienine (d18:2/22:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Behenoyl sphingomyelin (d18:1/22:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Sphingomyelin (d17:2/16:0, d18:2/15:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Sphingomyelin (d18:1/19:0, d19:1/18:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Sphingomyelin (d18:1/24:1, d18:2/24:0)*, Sphingolipid Metabolism	Placebo n3 LCPUFA	−0.1	
Sphingomyelin (d18:2/21:0, d16:2/23:0)*, Sphingolipid Metabolism	Placebo	−0.1	

Table 1 (continued)

Compound, Sub-pathway	Treatment	Effect size	Metabolism
Eicosanodioate (C20-DC), Fatty Acid, Dicarboxylate	n3 LCPUFA	−0.1	
	Placebo		
Sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*, Sphingolipid Metabolism	n3 LCPUFA	0.1	
	Placebo		
Sphingomyelin (d18:2/23:1)*, Sphingolipid Metabolism	n3 LCPUFA	−0.1	
	Placebo		
Stearoyl sphingomyelin (d18:1/18:0), Sphingolipid Metabolism	n3 LCPUFA	−0.1	
	Placebo		
1-(1-Enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)*, Plasmalogen	n3 LCPUFA	−0.1	
	Placebo		
Ceramide (d18:1/17:0, d17:1/18:0)*, Ceramides	n3 LCPUFA	0.1	
	Placebo		
Eicosenoate (20:1), Long Chain Fatty Acid	n3 LCPUFA	−0.2	
	Placebo		
Erucate (22:1n9), Long Chain Fatty Acid	n3 LCPUFA	−0.2	
	Placebo		
Glycerophosphoglycerol, Glycerolipid Metabolism	n3 LCPUFA	−0.2	
	Placebo		
Glycosyl ceramide (d18:1/23:1, d17:1/24:1)*, Ceramides	n3 LCPUFA	0.1	
	Placebo		
Glycosyl ceramide (d18:2/24:1, d18:1/24:2)*, Ceramides	n3 LCPUFA	−0.3	
	Placebo		
Glycosyl-N-behenoyl-sphingadienine (d18:2/22:0)*, Ceramides	n3 LCPUFA	−0.2	
	Placebo		
Myristoleate (14:1n5), Long Chain Fatty Acid	n3 LCPUFA	−0.2	
	Placebo		
Nonadecanoate (19:0), Long Chain Fatty Acid	n3 LCPUFA	−0.1	
	Placebo		
	n3 LCPUFA	−0.2	

3.6. Breastfeeding and child metabolite levels at age 6 months

Many metabolites can be transferred from mothers to children via the breastmilk and breastfeeding status may therefore influence the child metabolome. To disentangle this, we investigated the association between duration of exclusively breastfeeding and levels of each of the main metabolites driving the PLS-DA model. We used the exclusively breastfeeding duration rather than the total breastfeeding duration, since we consider this a better estimate of breastfeeding exposure. However, in order to exclude possible differences due to any breastfeeding at the time of sampling, we repeated the analysis using the difference in days between the sampling date and the end of any breastfeeding. No substantial differences were observed. The results from these correlation models are depicted in Figure SF7 (in the Supplementary Material), showing that the levels of all the lipid related compounds (long chain fatty acids, sphingolipids, lysophospholipids, ceramides, carnitines, etc) are positively associated with exclusive breastfeeding duration in both the n-3 LCPUFA and placebo strata; i.e. increasing levels with increasing breastfeeding duration independent of supplementation status. A few of the metabolites showed inverse associations with breastfeeding such as xanthurenic acid, 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4) and eicosanodioic acid (C20-DC).

To further explore the role of breastfeeding, we used the intercept from the metabolite vs. breastfeeding length models to estimate metabolite levels for a breastfeeding length of 0 days, which might mainly reflect the in-utero metabolic changes. We thereafter investigated differences in metabolite levels for an estimated breastfeeding length of 0 days in the n-3 LCPUFA vs. placebo group and compared that to the same difference at time of sampling; i.e. 6 months. These results are depicted in Fig. 3, and the estimated 0 days of breastfeeding results only showed a significant difference for sphingomyelin (C37n1) and (C33n1), palmitoyl-arachidonoyl-glycerol, hydroxy-CMPF, N-behenoyl-sphingadienine, and 3-(4-hydroxyphenyl) lactate between n-3 LCPUFA and placebo. All these differences, except for hydroxy-CMPF, were no longer present at 6 months of age, suggesting that what we observe is mainly mediated by the breastfeeding. However, these results should be interpreted with caution, due to the uncertainty of the estimation.

3.7. High-dose vitamin D intervention and child metabolomics at age 6 months

As 623 of the pregnant women in the n-3 LCPUFA trial also participated in high-dose vs. standard dose vitamin D supplementation DB-RCT with a nested 2×2 factorial design, we also investigated whether the vitamin D supplementation affected the child's metabolite levels at age 6 months. No effect of the vitamin D supplementation was observed on the child's metabolites levels using univariate linear modelling (Figure SF8 in the Supplementary Material).

3.8. The n-3 LCPUFA metabolome and risk of asthma at age 5

We have previously demonstrated a 30.7% relative reduction in the risk of developing asthma in the first 5 years of life in children, whose mothers were supplemented with n-3 LCPUFA during pregnancy compared to placebo [3]. As we hypothesized that part of this risk reduction could be mediated via the child metabolome, we tested possible associations between the components of the PLS-DA model and asthma development by age 5.

We initially found a significant association between the second latent score (sLV2) of the PLS-DA model and risk of asthma using a Cox regression analysis: hazard ratio (HR) per std. sLV2 increase: 0.80, 95% CI 0.65–1.26, $p = .02$ [Wald test]. In order to better disentangle the n-3 LCPUFA supplementation effect from the asthma association, we rotated the PLS-DA model to capture the asthma mediated direction in the first component, leaving the supplementation effect in component two to five. In Fig. 4, a Kaplan-Meier curve shows the risk of developing asthma in the first 5 years of life in children with LV1 score from the rotated PLS-DA model (rsLV1) divided into tertiles. The curve shows that children with a low rsLV1 n-3 LCPUFA-like metabolome score, i.e. a metabolome which is opposite to the n-3 LCPUFA metabolome, have an increased risk of developing asthma irrespective of supplementation group: HR 0.76, (0.64–0.90), $p = .002$ [Wald test], with a change of one standard deviation, i.e. lower risk from increasing n-3 LCPUFA-like metabolome. Stratifying the analysis by the n-3 LCPUFA supplementation, we observed a significant association in the placebo strata but not in the n-3

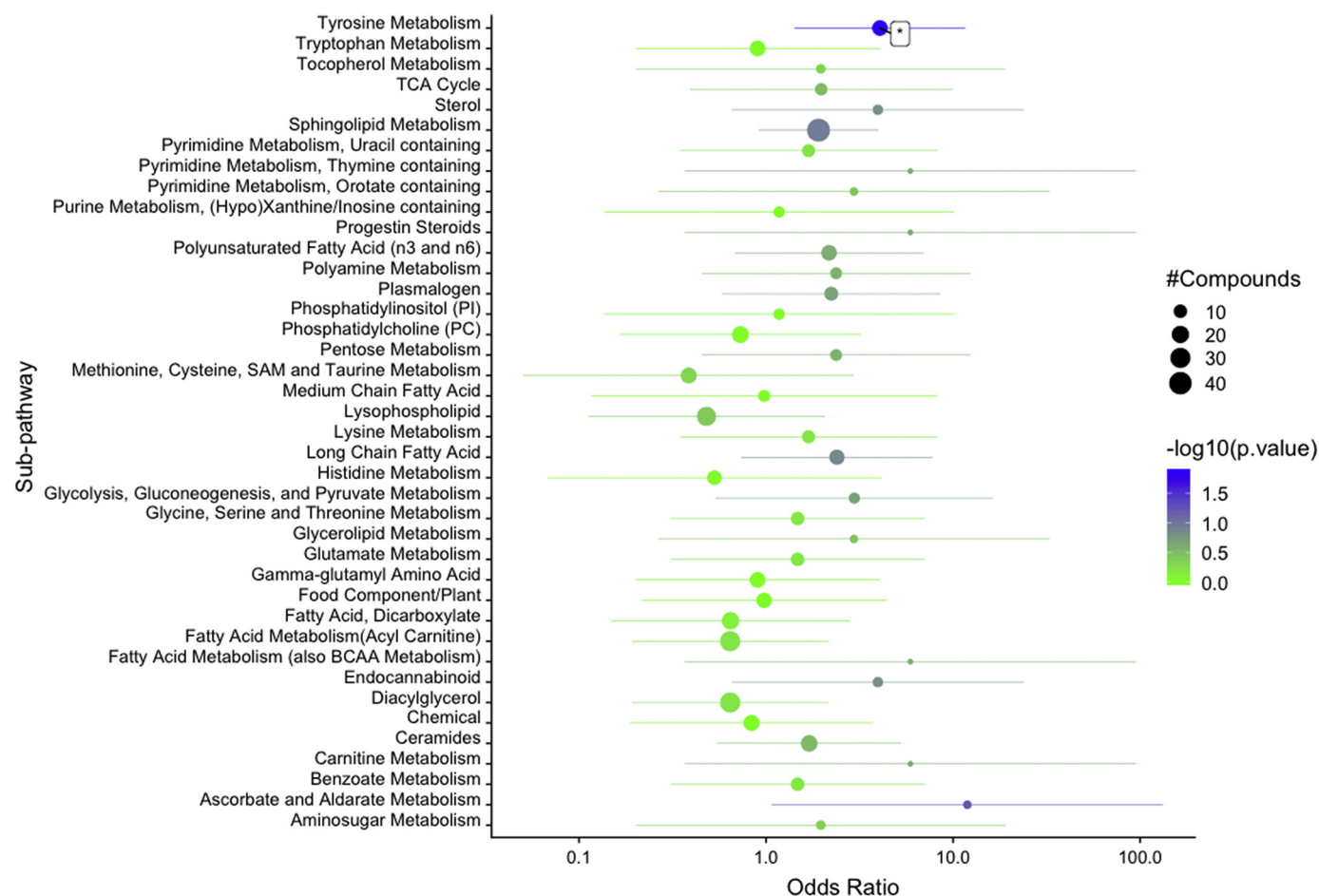


Fig. 2. Results from the enrichment analysis using Fisher's exact test. The odds ratio from each sub-pathway and the corresponding CI at 95% are colored based on the $-\log_{10}(p\text{-value})$, whereas the size of the colored circles is based on the number of compounds (from the PLS-DA) in the corresponding sub-pathway. (*): $p\text{-value} < .05$.

LCPUFA strata, which is probably due to a saturation effect of the supplementation on the metabolome in the n-3 LCPUFA group: per std. rLV1, $HR_{\text{placebo}} 0.65$, (0.50–0.85), $p = .002$ [Wald test] and $HR_{\text{LCPUFA}} 0.89$, (0.69–1.15), $p = .38$ [Wald test], respectively, see Fig. 5.

As we observed influence on the child metabolome from both FADS2 genotype and breastfeeding length, we adjusted the model for these parameters. From such sensitivity analysis we did not observe dependency from either exclusive breastfeeding duration or FADS2 genotype on the relationship between rLV1 and asthma risk: adjusted HR 0.75 (0.63–0.91), $p = .003$ [Wald test]. Furthermore, we also tested whether adjustments either for current breastfeeding at the time of sampling and total breastfeeding duration would influence the association with asthma development, but no influence was found.

Finally, we investigated whether the asthma-protective effect from the n-3 LCPUFA supplementation during pregnancy was mediated via the metabolome by using rLV1 as a proxy of the n-3 LCPUFA-associated metabolome by age 6 months. The result from this mediation analysis showed that 24% of the asthma-protective effect is mediated through the metabolome (see Table S4).

4. Discussion

4.1. n-3 LCPUFA supplementation during pregnancy and the child metabolome

We observed a decrease in metabolite levels linked to the n-6 pathway in children, whose mothers received fish oil during pregnancy. It has previously been observed that a dietary increased intake of 20:5n-3 eicosapentaenoic acid (n3-EPA) and 22:6n-3 docosahexaenoic acid

(n3-DHA) leads to a decrease of arachidonic acid (20:4n-6, AA) biosynthesis due to inhibition of $\Delta 6$ desaturation of linoleic acid (18:2n-6) [17,18]. In line with this, we observed decreased levels of AA in the n-3 LCPUFA supplementation group along with a reduction in levels of the AA precursor dihomo-linolenic acid (20:3n-6). We also registered lower levels of AA esterified glycerophospholipids, possibly due to acylation affinity for AA compared to the subsequent products in the pathway, which also undergo peroxisomal beta-oxidation, partially leading back to AA [19]. Finally, we observed decreased levels in the n-3 LCPUFA group of the end product in the n-6 pathway, docosapentaenoic acid (22:5n-6), which is probably due to a lack of AA as substrate as well as for the plasmalogens and diacylglycerols containing AA in the sn-2 position. Overall, it might be speculated that the n-3 LCPUFA supplementation led to some of the AA being replaced by EPA or DHA in the structural lipids; however, in our untargeted metabolomics analysis we did not observe an increase in the n-3 LCPUFAs or in their phospholipids.

With regard to lipids, we observed that the n-3 LCPUFA supplementation induced changes in the child metabolome with a decrease in levels of ceramides and sphingolipids containing 18:0 and 22:0 FAs. It can be speculated that there is an influence in the fatty acid metabolism from the stearic acid (18:0) to docosanoic acid (22:0) formation and a successive decrease in phospholipid formation.

The n-3 LCPUFA supplementation led to decreased levels of xanthurenic acid, which is a catabolic product from tryptophan in the kynurenine pathway. Xanthurenic acid is produced by the transamination of 3-hydroxykynurenine, a compound that can generate free radicals and apoptosis [20]. The indoleamine 2,3-dioxygenase 1 and 2 (IDO1/IDO2) enzymes are the main rate limiting enzymes activating

Table 2

Associations between FADS2 polymorphism (rs1535) and child n-6 pathway-related metabolite levels stratified by the intervention (n-3 LCPUFA supplement or placebo). Reported p-values are from “Wald test”.

Metabolite, Sub-pathway	Intervention	Mother's genotype			Child's genotype		
		beta-Estimate	p-value	CI	beta-Estimate	p-value	CI
1-(1-Enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, Plasmalogen	Placebo	−0.29	0.01	−0.51 −0.08	0.07	0.52	−0.15 0.29
1-(1-Enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*, Plasmalogen	LCPUFA	−0.10	0.32	−0.3 0.1	0.01	0.93	−0.2 0.22
1-(1-Enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*, Plasmalogen	Placebo	−0.34	<0.001	−0.51 −0.18	0.09	0.29	−0.08 0.27
1-(1-Enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*, Plasmalogen	LCPUFA	−0.06	0.53	−0.25 0.13	−0.01	0.90	−0.21 0.19
1-Arachidonoyl-GPC (20:4n6)*, Lysophospholipid	Placebo	−0.24	0.02	−0.45 −0.04	−0.05	0.66	−0.26 0.17
1-Arachidonoyl-GPC (20:4n6)*, Lysophospholipid	LCPUFA	−0.13	0.16	−0.32 0.05	−0.12	0.24	−0.32 0.08
1-Arachidonoyl-GPE (20:4n6)*, Lysophospholipid	Placebo	−0.19	0.07	−0.4 0.02	0.17	0.12	−0.05 0.39
1-Arachidonoyl-GPE (20:4n6)*, Lysophospholipid	LCPUFA	−0.17	0.09	−0.36 0.03	−0.01	0.95	−0.21 0.2
1-Palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6), Phosphatidylcholine (PC)	Placebo	−0.43	<0.001	−0.62 −0.24	0.08	0.45	−0.12 0.28
1-Palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6), Phosphatidylcholine (PC)	LCPUFA	−0.05	0.61	−0.25 0.15	−0.22	0.04	−0.43 −0.01
1-Stearoyl-2-arachidonoyl-GPC (18:0/20:4), Phosphatidylcholine (PC)	Placebo	−0.34	<0.001	−0.53 −0.15	0.00	0.97	−0.19 0.2
1-Stearoyl-2-arachidonoyl-GPC (18:0/20:4), Phosphatidylcholine (PC)	LCPUFA	−0.05	0.61	−0.25 0.15	−0.17	0.12	−0.39 0.04
1-Stearoyl-2-arachidonoyl-GPI (18:0/20:4), Phosphatidylinositol (PI)	Placebo	−0.23	0.03	−0.45 −0.02	0.09	0.40	−0.13 0.32
1-Stearoyl-2-arachidonoyl-GPI (18:0/20:4), Phosphatidylinositol (PI)	LCPUFA	−0.13	0.22	−0.33 0.08	−0.05	0.67	−0.26 0.17
Arachidonate (20:4n6), Polyunsaturated Fatty Acid (n3 and n6)	Placebo	−0.17	0.11	−0.39 0.04	0.07	0.54	−0.15 0.29
Arachidonate (20:4n6), Polyunsaturated Fatty Acid (n3 and n6)	LCPUFA	−0.09	0.35	−0.28 0.1	0.02	0.84	−0.18 0.22
Dihomo-linolenate (20:3n3 or n6), Polyunsaturated Fatty Acid (n3 and n6)	Placebo	−0.17	0.10	−0.37 0.03	0.17	0.11	−0.04 0.38
Dihomo-linolenate (20:3n3 or n6), Polyunsaturated Fatty Acid (n3 and n6)	LCPUFA	−0.07	0.49	−0.25 0.12	0.15	0.14	−0.05 0.35
Docosapentaenoate (n6 DPA; 22:5n6), Polyunsaturated Fatty Acid (n3 and n6)	Placebo	−0.24	0.04	−0.46 −0.01	0.22	0.07	−0.02 0.45
Docosapentaenoate (n6 DPA; 22:5n6), Polyunsaturated Fatty Acid (n3 and n6)	LCPUFA	−0.01	0.87	−0.19 0.16	−0.01	0.96	−0.2 0.18
Palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]*, Diacylglycerol	Placebo	−0.30	0.01	−0.52 −0.08	0.19	0.10	−0.04 0.42
Palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]*, Diacylglycerol	LCPUFA	−0.13	0.11	−0.3 0.03	0.03	0.77	−0.15 0.2
Stearidonate (18:4n3), Polyunsaturated Fatty Acid (n3 and n6)	Placebo	−0.09	0.48	−0.33 0.16	0.03	0.84	−0.23 0.28
Stearidonate (18:4n3), Polyunsaturated Fatty Acid (n3 and n6)	LCPUFA	−0.02	0.81	−0.18 0.14	−0.05	0.54	−0.22 0.12
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [1]*, Diacylglycerol	Placebo	−0.30	0.01	−0.51 −0.09	−0.10	0.36	−0.32 0.12
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [1]*, Diacylglycerol	LCPUFA	−0.21	0.02	−0.39 −0.04	−0.16	0.09	−0.34 0.02
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [2]*, Diacylglycerol	Placebo	−0.31	<0.001	−0.52 −0.09	−0.08	0.50	−0.3 0.15
Stearoyl-arachidonoyl-glycerol (18:0/20:4) [2]*, Diacylglycerol	LCPUFA	−0.17	0.06	−0.35 0.01	−0.04	0.70	−0.23 0.16

beta-Estimates are per copy of the G minor allele in rs1535. All the metabolites were autoscaled prior to the analysis.

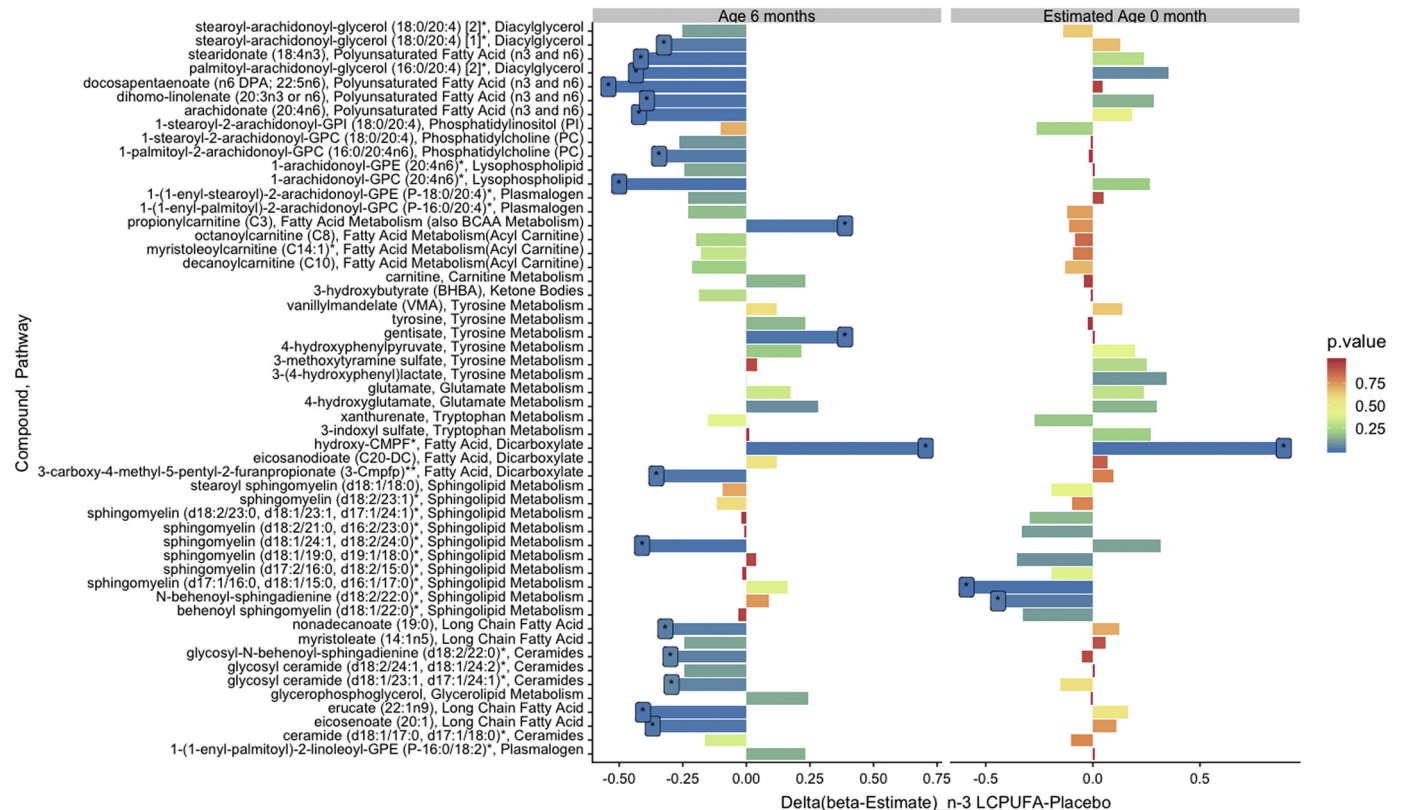


Fig. 3. Difference between metabolite levels in the n-3 LCPUFA and placebo group at estimated age 0 (estimated from the intercept) and at age 6 months. Bars show the difference in the beta-estimates and they are color coded based on the p-values. The metabolites were autoscaled prior analysis. Reported p-values are from “Wald test” (*): p-value < .05.

tryptophan catabolism through the kynurenine pathway. IDO expression is induced by lipopolysaccharides and cytokines, especially by interferon gamma (IFN- γ), that acts on T-cells and promotes differentiation and apoptosis via the T-cell receptor [21]. Our findings suggest that n-3 LCPUFA supplementation has an impact on the regulation of IDO expression, that may be caused by anti-inflammatory activity and reduced IFN- γ levels, leading to a reduction in tryptophan catabolism in the kynurenine pathway.

Medium- and short-chain acyl-carnitine were also affected with lower levels in the n-3 LCPUFA group. Acyl-carnitines are reversibly formed by the reaction of the acyl-CoA esters with carnitine, a process catalyzed by carnitine acyl-transferase. This mechanism takes place to transport the FAs with >14 carbons into the mitochondrial membrane for their subsequent beta-oxidation [22]. The concentration of acyl-carnitines in plasma also reflects the nutritional state and the contribution from other tissues, in particular, medium and long-chain acyl-carnitine are directly derived from the fatty acid metabolism. It can be speculated that n-3 LCPUFA supplementation may influence the beta-oxidation in the mitochondria, probably increasing the beta-oxidation, and therefore limiting the accumulation of intermediate lipid products. We also observed an increase in carnitine biosynthesis, with both carnitine and its precursor trimethyllysine being enhanced in the n-3 LCPUFA group.

In the n-3 LCPUFA group, we also observed an increase in tyrosine catabolism and its derived catecholamines breakdown products. Specifically, we observed an increase in vanillylmandelic acid, the main inactive catabolic product of norepinephrine and epinephrine. These catecholamines are produced from dopamine and act as hormones and neurotransmitters and are synthesized in the brain and in the adrenal gland. The other upregulated compounds in this pathway, 3-(4-hydroxyphenyl)lactate and 3-methoxytyramine sulfate, are metabolites originating from dopamine catabolism. It has been shown that rats chronically deficient in n-3 FAs have effects on the monoaminergic system during development with different effect in different regions of the cortex [23]. Furthermore, rats receiving a n-3 LCPUFA-enriched diet that was poor in linoleic acid (18:2n-6) [24] showed significant differences in the FA composition in the cerebral membranes with higher levels of docosahexaenoic acid (22:6n-3), that is the most abundant n-3 FA in

the brain, lower levels of AA and higher levels of dopamine. It has also been shown in mice that fish oil supplementation upregulates the expression of the mitochondrial uncoupling protein 1 in the brown and beige adipose tissue, which affects fat accumulation. The brown adipose tissue activity is primarily regulated by the sympathetic nervous system, which releases norepinephrine that successively binds the beta-adrenergic receptors and promotes the expression. This aligns with observations of increased release of norepinephrine in the urine of mice receiving fish oil supplementation [25] and may partly explain the relationship between the n-3 LCPUFA supplementation and upregulation of the tyrosine pathway.

The n-3 LCPUFA supplementation was also associated with an increase in glutamate, 4-hydroxy-glutamate, which is a byproduct of 4-hydroxy-2-oxoglutarate, and alpha-ketoglutarate which is a deamination product and precursor of glutamate. Glutamate is a non-essential amino acid that can be synthesized from alpha-ketoglutarate, glutamine and pyroglutamic acid. Glutamate is involved in several pathways both as substrate and product and is also an excitatory neurotransmitter [26]. So far, no direct associations have been found between n-3 LCPUFA intake and the glutamate metabolism.

Hydroxy-CMPF and 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid are metabolites, which in humans come from beta-oxidation and omega-oxidation of furan FAs. Furan FAs are formed in plants, bacteria, and algae and they accumulate in the tissues of fish and crustaceans [27]. In humans, the uptake of the furan FAs is through food such as vegetables and vegetable oils, fish and fish oil. However, there is a hypothesis of de novo synthesis of CMPF by the gut microbiota [27]. We observed a much higher level of hydroxy-CMPF in the children, whose mothers received fish oil-derived n-3 LCPUFA compared to placebo, which is presumably directly related to the fish oil supplementation and therefore serves as a biological validation of the metabolomics dataset.

4.2. FADS2 genotype and n-3 LCPUFA supplementation

The amount of n-3/n-6 LCPUFA is dependent on food or supplementation but also on the activity of the $\Delta 5$ - and $\Delta 6$ -desaturase enzymes, which converts the essential FAs into LCPUFAs. The mother's FADS2

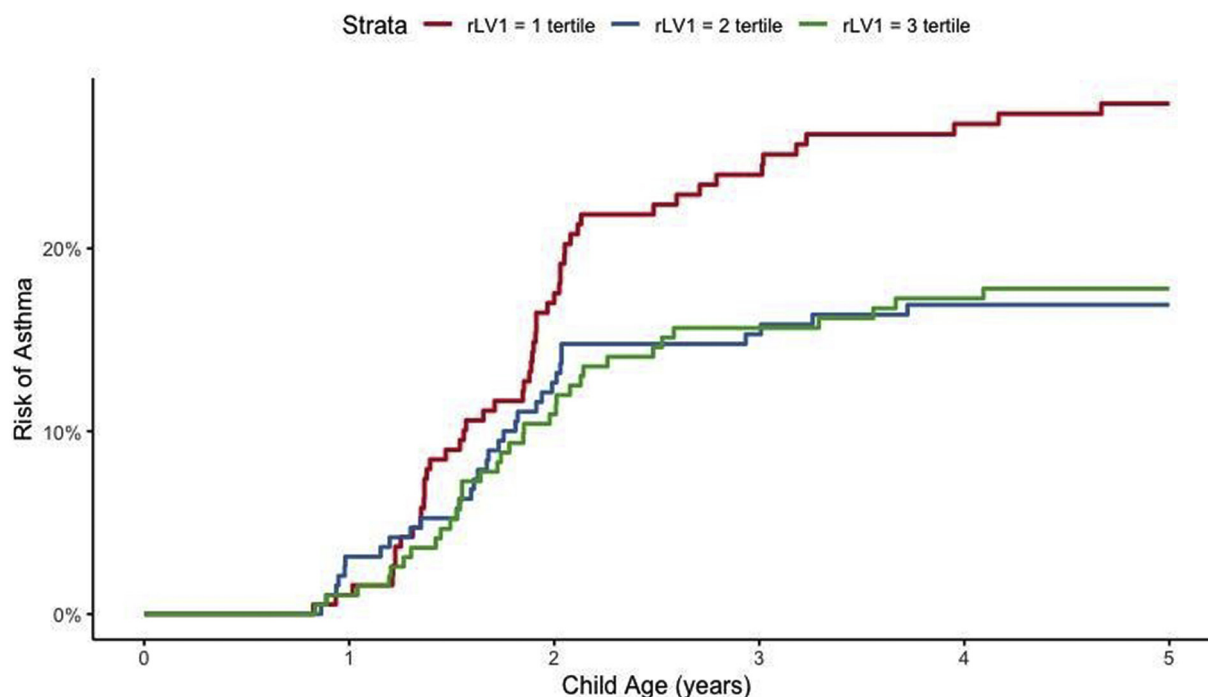


Fig. 4. Kaplan-Meier curve of the first component of the rotated PLS-DA model (rLV1) divided into tertiles and the risk of developing asthma by age 5.

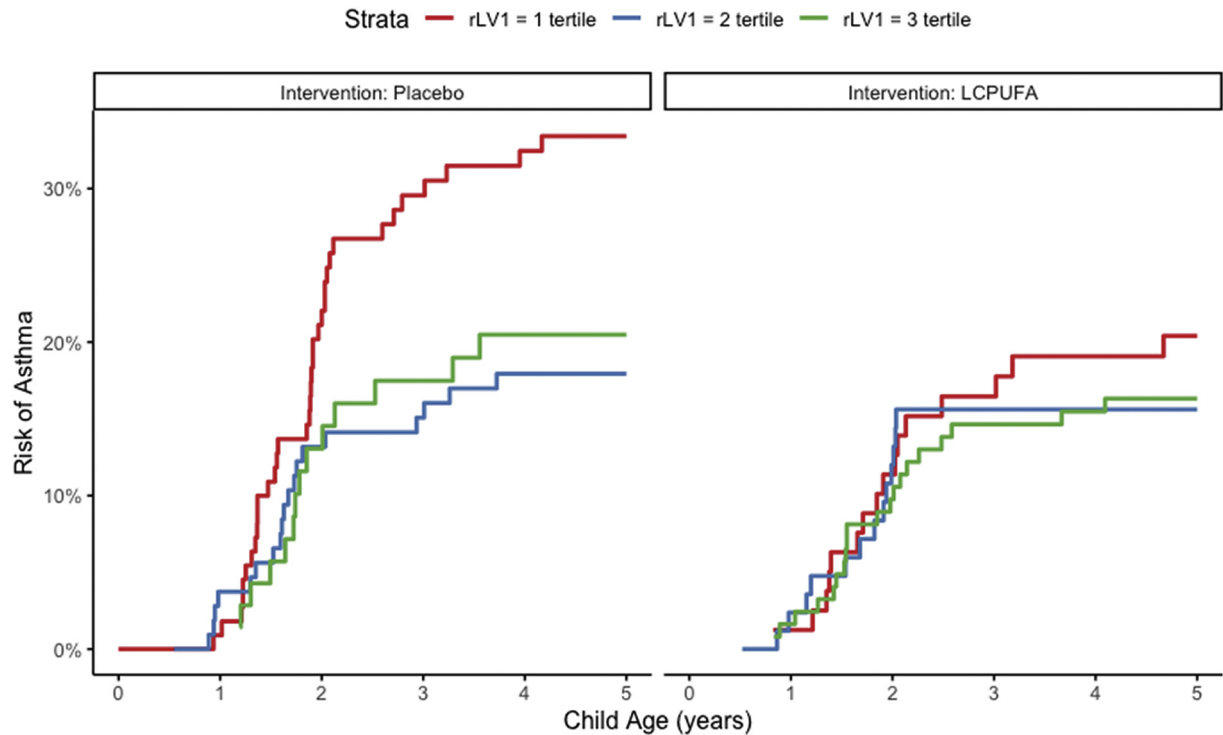


Fig. 5. Kaplan-Meier curve of the first component of the rotated PLS-DA model (rLV1) divided into tertiles and the risk of developing asthma by age 5, stratified by intervention.

genotype was strongly associated with levels of metabolites in the n-6 pathway whereas the child's genotype was not. It might be that the child's metabolite levels in this pathway are primarily a reflection of the maternal levels via breastfeeding [28]. Furthermore, the quasi significant association of the main FAs is in line with previous studies showing that the minor allele is associated with lower enzyme activity and therefore lower levels of fatty acid products [28,29], in our case AA, n-6 DPA and 20:3n6. The reason why we observe a stronger significance for the AA derived products could be due to a better sensitivity for the compounds further down the cascade process, but still directly derived from AA. The effect of the genotype is predominant in the placebo strata compared to the fish oil strata, probably due to the effect of the supplementation, which affects the activity of the desaturase enzyme and probably masking and compensating for the genotype effect.

4.3. Breastfeeding effect on the child metabolome

We observed a strong influence of breastfeeding duration on metabolites in both the n-3 LCPUFA and placebo strata and used this information to address whether the n-3 LCPUFA supplementation induced a pre- or post-natal programming effect on the child metabolome. Our results suggest that the n-3 LCPUFA supplementation effects on the child's metabolome are mainly originating from the composition of the mother's breast milk. However, most of the differences in metabolite levels between n-3 LCPUFA and placebo for estimated 6 months and 0 days of breastfeeding have the same directionality and the fit of the regression model may suffer from fewer cases of children not being exclusively breastfed for a long period of time, thus affecting the estimation at 0 days. We assume that breastfeeding duration plays an important role in enhancing the level of n-3 LCPUFAs in the children, but at the same time, we speculate that some of the compounds seem to be transferred through the placenta or are altered due to prenatal changes, even though we need metabolomics profiles of the children's plasma at birth to verify this assumption. Further, assessments of maternal metabolomics profiles would enable us to disentangle whether the n-3 LCPUFA supplementation during pregnancy lead to sustained changes in mother's metabolite levels, which could be transferred to the child

via the breastmilk. If it is correct that most of the metabolite differences in relation to n-3 LCPUFA supplementation are a result of breastfeeding, it suggests that breastfeeding may accentuate the effects of prenatal n-3 LCPUFA supplementation.

4.4. n-3 LCPUFA-associated metabolome and asthma-protective effect

We aimed to relate the effect of the n-3 LCPUFA supplementation on the child metabolome to the observed asthma-protective effect in our DB-RCT to disentangle the underlying biochemical mechanisms. Our analysis suggested that the n-3 LCPUFA supplementation associated metabolome mediated 24% of the asthma-protective effect.

Part of the mediation effect on asthma may be explained by the down-regulation of the n-6 LCPUFAs and their esterified compounds. Particularly, AA is one of the most abundant LCPUFAs in the cells involved in the inflammatory process leading to asthma symptoms. AA is a substrate for cyclooxygenase, lipoxygenase and cytochrome P450 enzymes to produce eicosanoids, which are potent mediators of airway inflammation. Thus, the prostaglandins 2-series (PGE2) are involved in the induction of the proinflammatory interleukin 6 (IL-6) [30] and increased levels of the leukotrienes LTC4 and LTD4 resulting in bronchoconstriction, increased mucus secretion, and airway hyperreactivity, that leads to airflow obstruction in asthma patients [31]. In line with this, a case-control study among adults with asthma have previously shown increased circulating AA levels [32].

We also observed an association of the n-3 LCPUFA supplementation on the glutamate pathway. Glutamate is involved in several pathways, including the gamma-glutamyl cycle in the liver, where glutathione is produced from glutamate cysteine and glycine [33]. Glutathione is an antioxidant compound and lower levels of glutamic acid, glycine and tyrosine have previously been demonstrated in asthmatic children [34], thus connecting childhood asthma with a decreased antioxidant defense. In our study, we observed that children, whose mother received n-3 LCPUFA had higher levels of tyrosine, glutamic acid as well as dimethylglycine and sarcosine, which are precursors of glycine, thereby linking the n-3 LCPUFA supplementation to an improved antioxidant system, which may have played an asthma-protective role. In this

study we demonstrate that n-3 LCPUFA supplementation with fish oil during pregnancy significantly associates with the offspring metabolome by age 6 months. The observed effects were primarily a decrease in metabolites in the n-6 LCPUFA and tryptophan pathways, as well as an increase in the tyrosine and glutamate pathways. We further show that the fish oil metabolomic profile was associated with a reduced risk of asthma by age 5 and that these biochemical alterations mediated one fourth of the asthma-protective effect of the supplementation. These findings provide new important insight into the effects of dietary n-3 LCPUFA supplementation and the pathogenesis of childhood asthma.

Authors contributions

H.B. had full access to all of the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

H.B. and B.L.C. contributed equally to the manuscript.

Study concept design and acquisition: H.B., B.L.C., K.B., J.S., M.A.R.

Statistical Analysis: D.R., M.A.R.

Interpretation of data: All authors.

Drafting of Manuscript: D.R.

Critical revision of the manuscript for important intellectual content: All authors.

Source of funding

All funding received by COPSAC is listed on www.copsac.com. The Lundbeck Foundation (Grant no R16-A1694); The Ministry of Health, Denmark (Grant no 903516); Danish Council for Strategic Research (Grant no 0603-00280B); The Capital Region Research Foundation have provided core support to the COPSAC research center. In addition, COPSAC and VDAART have received funding from NIH (Grant no. R01HL141826).

Governance

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Declaration of Competing Interest

All authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.07.057>.

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